Reinfection of Chancre-Immune Rabbits With Treponema Pallidum

I. Light and Immunofluorescence Studies

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Inoculation of infectious *Treponema pallidum* into the skin of chancre-immune rabbits results in a limited inflammatory response. Intact organisms are identifiable by immunofluorescence in the dermis of the infection site for 1–2 days. By Day 3 structurally intact *T pallidum* are seen localized in hair follicles, erector pili muscles, and cutaneous nerves, while inflammatory cells containing fluorescent (*T pallidum*) fragments are seen in the dermis. After Day 6 intact organisms are no longer found. It is proposed that hair follicles, erector pili muscles, and particularly nerves may provide relatively protected sites for *T pallidum*, and that *T pallidum* may migrate within nerves. Clearance of organisms from the infected site appears to be mediated by phagocytosis and digestion by macrophages as a result of an accelerated delayed hypersensitivity response, but antibody-mediated destruction and *T pallidum* migration may also be involved. (Am J Pathol 1985, 118:248–255)

IT HAS long been known that resistance to reinfection with *Treponema pallidum* occurs during the course of human and experimental syphilitic infection.[1-3] However, the mechanisms of resistance and the nature of the immunopathologic changes resulting from reinfection are still poorly understood. Much of our knowledge regarding the development of immunity during syphilis is based on immunologic and histologic studies in experimentally infected rabbits. In this model, the primary lesion of syphilis appears to be a manifestation of delayed hypersensitivity. Following injection into the testicle[4,5] or shaved skin[6] of nonimmune rabbits, there is a marked increase in *T pallidum* at the injection site until approximately 12–14 days after inoculation, when rapid clearing occurs. Clearance of infecting organisms occurs at a time when there is a specific systemic humoral and cellular immune response to *T pallidum*[7] and is associated with T-cell and macrophage infiltration of the infection site and hyperplasia of lymph nodes and spleen.[8] T-cell reactivity to *T pallidum* antigens can be demonstrated in the lymph nodes and spleen within the first week after infection, and rising antibody titers occur soon thereafter.[6,7] Destruction of infecting organisms primarily occurs by phagocytosis and digestion by macrophages.[9] Although most of the infecting organisms are eliminated during this response, a few escape immune destruction and persist in the tissues of infected animals in the absence of tissue lesions (latency).[1,3,6,10] Disease may be transferred with the use of tissue of animals in latency which demonstrate no evidence of infection other than persistent humoral and cellular immune reactivity.[1,2,11] The mechanism of persistence of viable organisms during latent syphilis remains a mystery. Proposed mechanisms include immune suppression, antigen modulation, or isolation of the organisms from immune attack in "protective niches."[1,3,6,12]

The present study was designed for determination of the fate of *T pallidum* injected into the skin of rabbits that had recovered from an active infection and were resistant to challenge (so-called "chancre immunity"[1-3]). The distribution of organisms was traced by immunofluorescence microscopy.

Supported by NIH Grant AI-19810.

Accepted for publication September 12, 1984.

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Materials and Methods

Animals

A total of 19 adult male New Zealand white rabbits were used. Thirteen of these rabbits were infected intratesticularly and had gross and microscopically proven syphilitic orchitis 6 months to 3 years prior to infection. All but 2 of the rabbits used were obtained from Rancho de Conejo, Vista, California; 2 noninfected control rabbits were obtained from Ray Nichols Rabbitry, Lumberton, Texas. All animals were fed antibiotic-free food and were maintained at 18–20 C.

Experimental Infection

T pallidum, Nichols strain, was originally provided by Dr. James N. Miller (UCLA) and has been subsequently passed in rabbits in our laboratory.[4,5] All rabbits were VDRL-nonreactive prior to initial infection. In order to study the early events during reinfection of immune rabbits, a series of five experiments as identified in Table 1 was performed. The biopsy times were selected to provide appropriate times after challenge for determining the fate of the injected organisms.

Skin sites were biopsied by oval incision under anesthesia at the times indicated in Table 1. At least one site was left for gross observations until sacrifice of the rabbit. The skin biopsies were bisected at the site of inoculation. One part was fixed in 10% phosphate-buffered formalin for staining with hematoxylin and eosin (H&E), and one in 95% ethanol, 1% acetic acid for immunofluorescence.[5] Tissue for light and fluorescence microscopy was processed as described previously.[4,5]

Results

Gross Development of Lesions

As previously described by a number of investigators,[1,2,13] the time of appearance of development of dermal syphilitic lesions in previously uninfected rabbits is dose-dependent. At the higher doses used in this study (2.5×10^7 to 2×10^8 per site), erythema and induration are first seen at 24 hours. This reaction expands to marked swelling and ulceration by Day 9–10. In chancre-immune rabbits, very slight erythema is seen 1 day after infection. At 2 and 3 days after infection, small areas of erythema and induration (less than 6–7 mm in diameter) are seen at most skin test sites, which last for 3–4 days. By Day 9 little or no gross manifestations of inflammation are present.

In the group of rabbits inoculated with 10^6 organisms, the development of gross lesions in the previously uninfected rabbits is delayed, so that, at termination of the experiment at Day 10, ulceration has not yet occurred. No gross lesions are seen in chancre-immune rabbits given injections of 10^6 organisms. The histologic description which follows will concentrate on lesion development in rabbits receiving the higher dosages of *T pallidum*. Similar, but less intense, reactions are observed in rabbits receiving 10^6 organisms.

Light Microscopy—Nonimmune Rabbits

The inflammatory response in the skin of nonimmune rabbits to the inoculation of *T pallidum* has been described extensively[2,6] and will be presented here briefly for comparison with the response of chancre-immune rabbits.

In nonimmune rabbits there is an early perivascular and diffuse dermal infiltrate of polymorphonuclear heterophilic cells with slight edema, particularly in the subepidermal connective tissue. By 24–48 hours the predominant cell type becomes mononuclear, but the degree of inflammation is slight. There is a progressive increase of round cells through Day 9, when there is extensive infiltration of the dermis, particularly perivascularly and around the hair follicles, with necrosis of the overlying epithelium. After Day 10, but with times that vary somewhat with individual rabbits, a primarily small round cell infiltrate is replaced by a larger monocyte population as well as increased fibroblastic activity, eventually progressing to scarring.[6]

Table 1—Experimental Design

Experiment	No. rabbits		Challenge dose (No. organisms)	Biopsy times	
	Nonimmune	Immune		Hours	Days
1	1	1	5×10^7	0.5, 1, 2, 4, 6	1, 2, 3
2	1	3	2×10^8	1, 6, 12	1, 2, 3, 10
3	2	2	10^6	1, 6	1, 2, 5, 10
4	0	3	10^8		1, 2, 3, 4, 5, 6, 7, 8, 9, 10
5	2	4	2.5×10^7	6	1, 2, 3

Light Microscopy – Immune Rabbits

Day 1

There is a mild perivascular mixed cellular infiltrate predominantly around very small venules. Nerves, arterioles, and larger veins are not involved. Polymorphonuclear heterophilic cells are in the majority (approximately 60%), although some venules are surrounded by mononuclear cells. A more diffuse polymorphonuclear heterophil infiltrate is present throughout the dermis in many areas with prominent subepithelial involvement and separation (edema) of collagen bundles (Figure 1A). There is less intense staining and apparent dissolution of collagen fibers immediately adjacent to vascular areas (mucoid degeneration).

Day 2

There is a more prominent perivascular infiltrate than at Day 1 consisting of approximately 30–50% heterophils and 50–70% mononuclear cells, with increased numbers of mononuclear cells, giving a cufflike appearance (Figure 1B). Marked endothelial swelling and margination of mononuclear cells in venules is now apparent. Cutaneous veins show perivenular infiltration of mononuclear cells and a few heterophils. A diffuse, mixed cellular infiltrate and edema are present in the deep dermis. Hair follicles and erector pili muscles are surrounded focally by heterophils.

Day 3

There is diffuse edema and infiltration with mononuclear cells throughout the subepithelial and deep dermal connective tissue (Figure 1C) as well as marked perivascular cuffing with endothelial swelling and margination. Small arterioles are essentially spared. The perivascular infiltrate is now almost entirely mononuclear. Further disruption of collagen in the perivascular areas is evident. There is infiltration of some hair follicles and erector pili muscles with mononuclear cells, which is more prominent than at Day 2. Many cutaneous nerves are lightly infiltrated with heterophils and mononuclear cells, but the structure of the nerve is still recognizable.

Day 4

Perivenular cuffing is prominent, and there is also a much more pronounced diffuse mononuclear cell infiltrate in the upper dermis, with separation of collagen bundles. Cutaneous nerves, erector pili muscles, and hair follicles are surrounded by and lightly infiltrated with mononuclear cells.

Day 5

Perivascular cuffing and more intense involvement of cutaneous nerves are obvious. There is a marked increase in diffuse mononuclear cell infiltrates, particularly in the upper dermis as well as more marked mononuclear infiltration around and in the nerves, erector pili muscles and hair follicles (Figure 1D). Heterophils now consitute less than 20% of inflammatory cells. In the upper dermis, the normal, parallel arrangement of collagen fibers is disrupted, and many fragmented cell nuclei are present. There are focal swirls of dense collagen in the dermis corresponding in distribution to areas of collagen swelling and dissolution seen earlier.

Day 6

The mononuclear cell infiltration is now much more delineated around nerves (Figure 1E). There is less diffuse infiltrate, and although still prominent, there is less inflammation in the skin appendages. Some perivascular infiltrates also contain RBCs. In prominent perivascular infiltrated areas, cutaneous nerves are surrounded by mononuclear cells. Nuclear debris and mononuclear infiltrates are still present diffusely in the upper dermis, whereas the lower dermis is relatively clear of diffuse infiltration, making the perivascular component more clearly demarcated. The amount of overall infiammatory cell infiltrate is less than seen on Days 4 and 5.

Day 7

There is still prominent perivascular and upper dermal infiltrate, but less pronounced than on Day 6 (Figure 1F). Hair follicles, erector pili muscles, and nerves show less intense inflammation. An apparent increase in fibrous tissue and fibroblastic activity can be seen around nerves.

Day 8 and 9

Some mononuclear and subepidermal infiltrate remains, but it is much less than seen earlier. The veins are cuffed by 3–4 cell layers of mononuclear cells. There is little or no diffuse dermal inflammation. Cutaneous nerves appear to be disrupted by residual mononuclear cells, and there are spaces between the nerve fibers that are not normally seen (Figure 1G).

Day 10

By Day 10, little inflammatory infiltrate remains. There are few mononuclear cells surrounding small veins. Cutaneous nerves and erector pili muscles are now essentially free of inflammatory cells. Although some disruption of nerve structure as seen on Day 9 is seen, it is less severe.

Immunofluorescence

The number of *T pallidum* organisms in the dermal connective tissue of nonimmune rabbits increases dur-

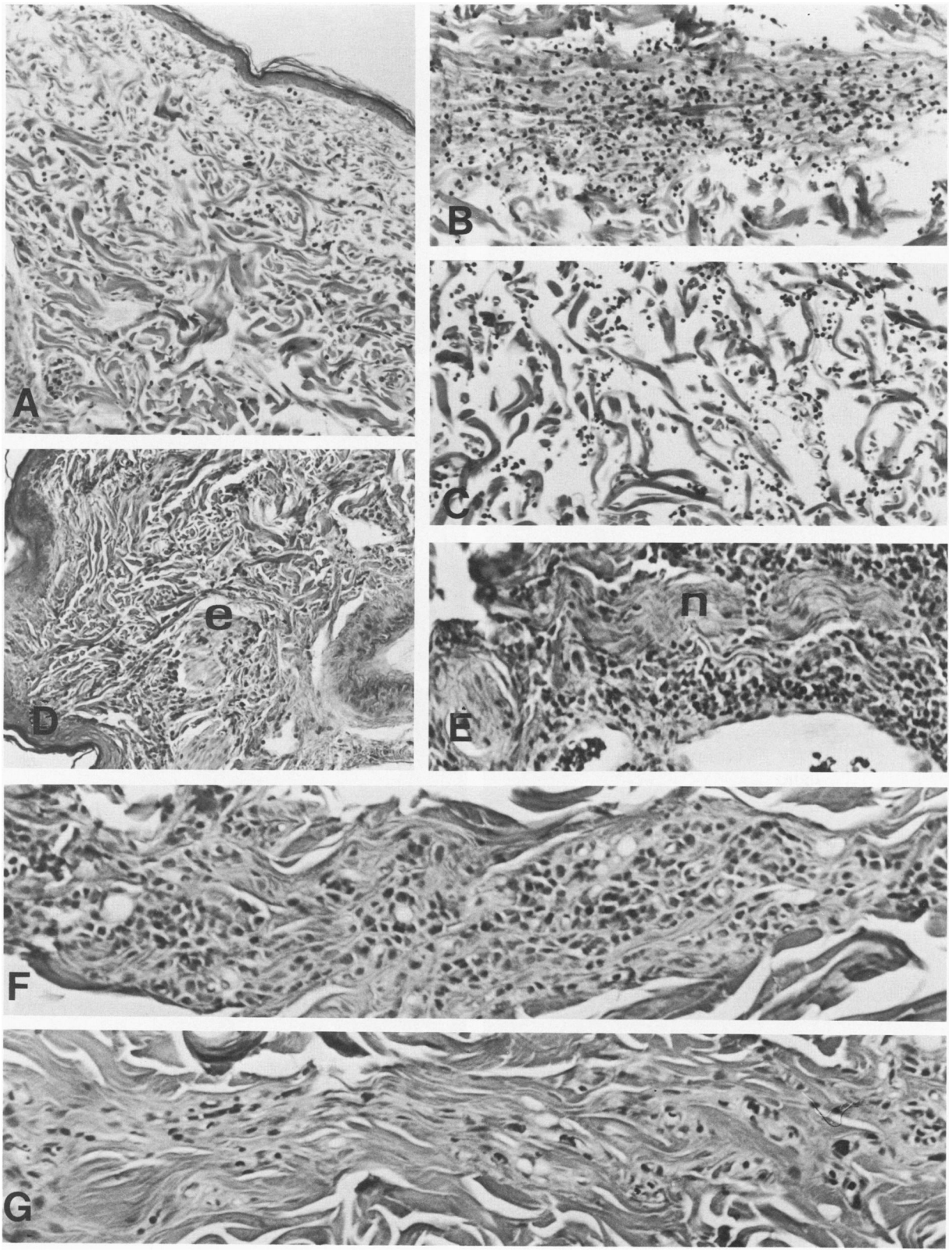

Figure 1—Light-microscopic evaluation of skin lesions in "chancre-immune" rabbits. (H&E) **A**—Day 1. Separation of collagen (edema) in dermis. (×160) **B**—Day 2. Mixed polymorphonuclear–monocyte vasculitis. (×160) **C**—Day 3. Dermal edema with mononuclear infiltrate. (×160) **D**—Day 5. Mononuclear infiltrate around erector pili muscles. (×160) **E**—Day 6. Perineural mononuclear infiltrate. (×320) **F**—Day 7. Mononuclear infiltrate in and around nerve. (×320) **G**—Day 9. Resolving neural inflammation. *e*, erector pili muscle; *n*, nerve.

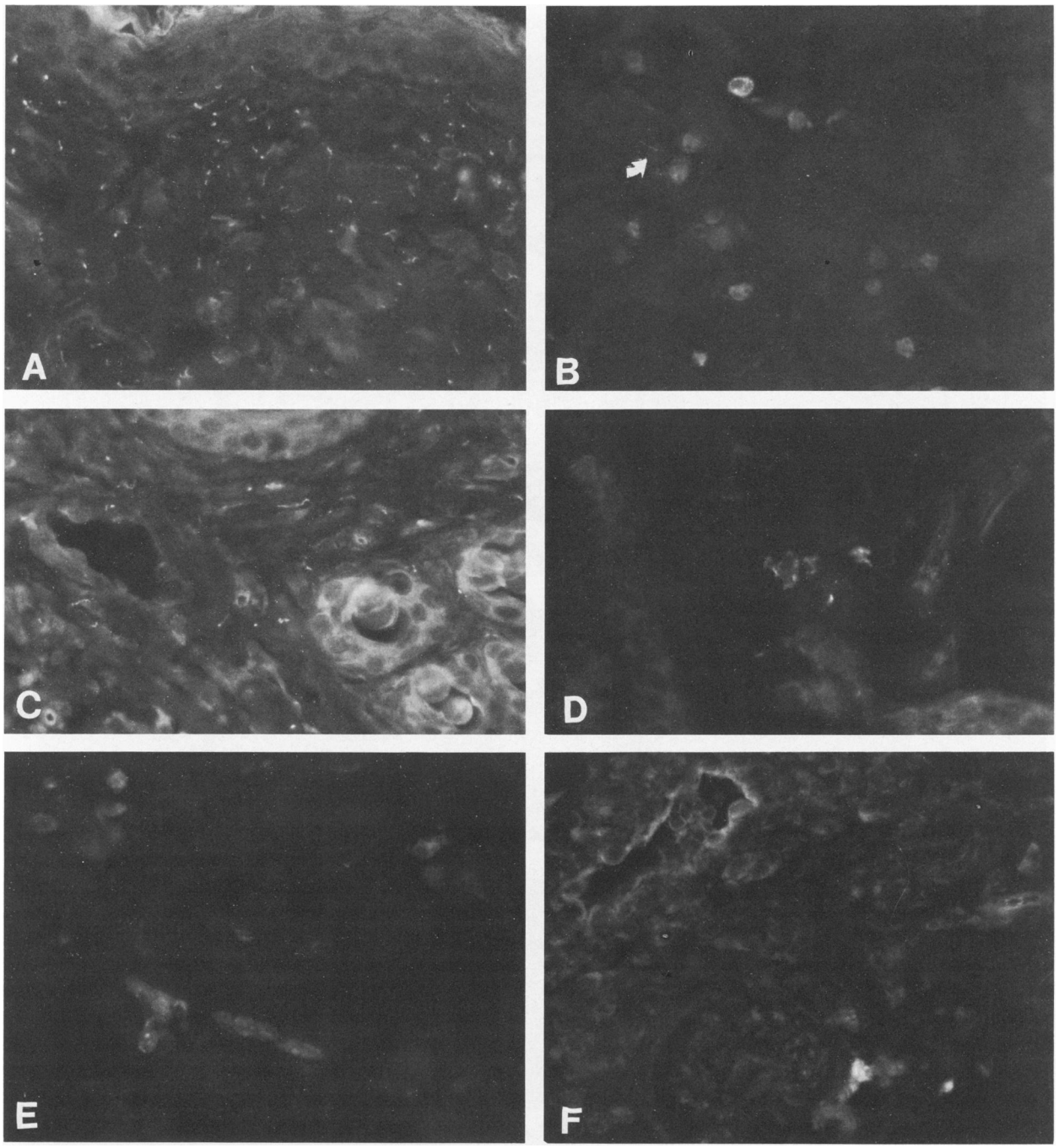

Figure 2—Immunofluorescence of *T pallidum* in skin of "chancre-immune" rabbits. (×400) **A**—Day 2. **B**—Day 4. **C**—Day 5. **D**—Day 7. **E**—Day 8. **F**—Day 9. Intact organisms may be seen from Day 1 through Days 5–6 (note the *arrow* in **B**). The number of organisms in the skin of chancre-immune rabbits decreases markedly after Day 3. Cells with fluorescent material become prominent on Days 4–8. By Day 9 little or no fluorescence is seen except for material in cutaneous nerves. These studies suggest that *T pallidum* organisms do survive in the skin of chancre-immune rabbits for 5–7 days but are eventually phagocytosed and digested by macrophages.

ing the first 9–10 days after primary inoculation.[6] Rapid clearance of organisms is seen from 10 to 14 days, after which time occasional organisms may be seen in dermal connective tissue. At the time of clearance, fluorescent material may be visualized in dermal mononuclear cells.[9]

In chancre-immune rabbits, organisms can be found concentrated in regions near the site of inoculation during the first 1–2 days. By Days 3–5 only rare organisms can be seen, and after Day 6 organisms are no longer seen (Figure 2). At Day 1, organisms are located in the dermis and appear structurally intact. On Day 2, many

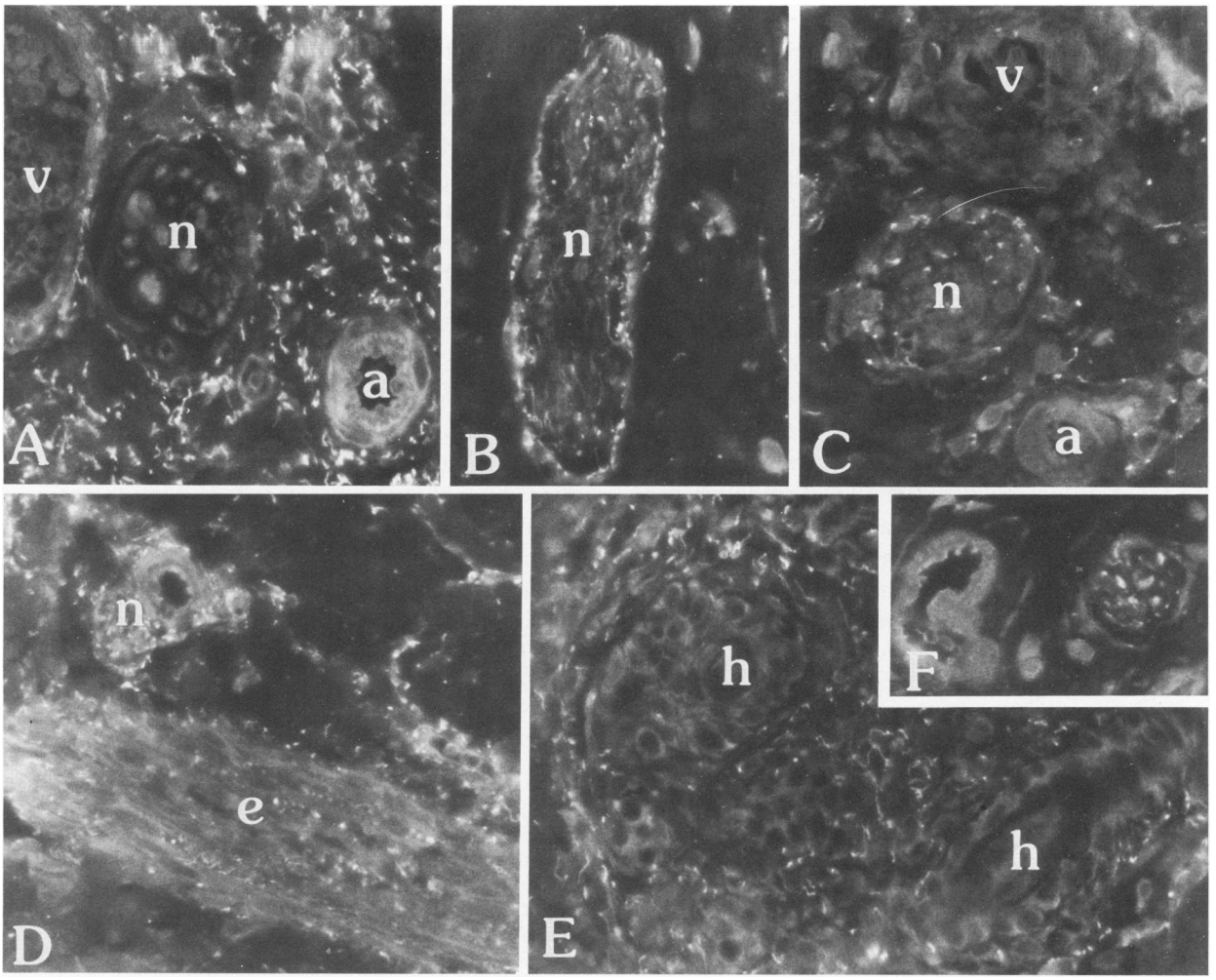

Figure 3—Preferential location of *T pallidum* in nerves, erector pili muscles, and hair follicles. **A**—Immune rabbit, 12 hours. **B**—Nonimmune rabbit, 48 hours. **C**—Immune, 72 hours. **D**—Immune, 72 hours. **E**—Immune, 72 hours. **F**—Immune, 72 hours. (×400). There is a diffuse spreading of organisms in the connective tissue of the dermis during the first 24 hours in both immune and nonimmune rabbits inoculated individually with 2.1×10^8 viable *T pallidum* organisms. The cutaneous nerves contain few, if any, organisms for the first 12 hours after injection, but organisms are seen prominently in cutaneous nerves and erector pili muscles and among cells in the hair follicles after 24 hours. This tendency is seen in both nonimmune and immune rabbits. At 72 hours relatively few organisms are seen in the connective tissue of the dermis of immune rabbits, but organisms are easily seen in nerves (**C**) and erector pili muscles (**D**) and focally among the cells of hair follicles (**E**). Note the perivascular cellular infiltrate in **C** and preferential localization of fluorescent *T pallidum* organisms in nerves in **B**, **C**, **D**, and **F**. The **inset** (**F**) shows a nerve containing fluorescent *T pallidum*. *a*, artery; *v*, vein; *n*, nerve; *e*, erector pili; *h*, hair follicle.

intact organisms are still visible in some areas, but perivascular and diffuse inflammatory cells containing fluorescent fragments are also evident. By Day 3, the diffuse and perivascular infiltrate has increased, and many inflammatory cells contain fluorescent fragments. Few intact organisms are seen. Fluorescent fragments are prominent in subepidermal and perivenular inflammatory cells. From Day 4 to Day 6 there is considerable variation in fluorescent staining among different sections. Intact *T pallidum* tend to be localized in the upper dermis, hair follicles, erector pili muscles, and nerve fibers (Figure 3). In most regions, however, intact organisms are few in number, and there is fluorescent material in inflammatory cells. After Day 6 no intact organisms are observed. Fluorescent fragments are occasionally seen in inflammatory cells in the upper dermis and in nerve fibers. After Day 9, no organisms or fluorescent fragments are seen.

Discussion

So-called chancre-immune rabbits are not completely resistant in reinfection by a second exposure to *T pallidum*. Chesney[1] and Magnuson and co-workers[13,14] have previously emphasized that immunity to reinfection in syphilis is relative and dose-dependent, inasmuch as asymptomatic infection can persist indefinitely following reinoculation of chancre-immune animals. In the present studies it is clearly shown that although there may be little or no gross lesion development in chancre-

immune rabbits challenged with *T pallidum*, intact organisms may be identified in the inoculation site for up to 3–5 days after injection, even when relatively small (10^6) numbers or organisms are injected.

In nonimmune rabbits, the number of organisms increases at the site of infection until a delayed hypersensitivity reaction occurs, usually about 10 days after inoculation. Clearance of organisms is mediated by infiltration by macrophages and digestion of organisms.[3,6,9]

The fate of organisms in chancre-immune rabbits appears to be similar, but the inflammatory reaction and clearance of *T pallidum* occur much more rapidly. However, other mechanisms in addition to delayed hypersensitivity could be operative. *T pallidum* may be killed directly or opsonized by antibody and complement.[15] However, antibody-mediated antimicrobial reactions typically produce a polymorphonuclear cell infiltrate, in contrast to the primarily mononuclear response seen in human and experimental syphilis. In addition, obvious coating of *T pallidum* by host immunoglobulin in lesions prior to or during clearance is not seen,[5,6,8] although some reaction of humoral antibody with *T pallidum* in situ may occur.[15] Migration of *T pallidum* from the site of infection undoubtedly accounts for part of the reduction in numbers observed locally.[2,11] Such dissemination was shown to occur in "immune" rabbits by Magnuson and Rosenau.[11] They demonstrated that *T pallidum* organisms capable of producing lesions in other rabbits were present in lymph nodes following reinoculation of chancre-immune rabbits which had been treated with penicillin to clear the initial infection.

The results presented in this study suggest that *T pallidum* may escape immune destruction by localizing in protective niches. This concept was stressed by Medici[12] in an attempt to explain latency. Latency refers to the presence of viable infectious organisms in the absence of detectable tissue lesions. *T pallidum* in the skin may temporarily escape immune attack by moving into the nerves, erector pili muscles, and hair follicles. The localization of inflammation around nerves, hair follicles, and erector pili muscles during the elimination phase of the inflammatory reaction indicates that these sites are not entirely "safe" for organisms during the cellular inflammatory response. It is also possible that *T pallidum* may actually reside inside cells.[16–22] In either case these "niches" could provide protected sites for *T pallidum* during active infection and latency.

Involvement of cutaneous nerves raises the possibility that *T pallidum* may pass to the central nervous system (CNS) by transmission along nerve fibers.[23,24] CNS transmission via nerves is believed to occur in herpes simplex infections.[25–27] Inflammation of nerve tissue is associated with the pathogenesis of tabes dorsalis and optic atrophy in neurosyphilis, similar to the neuritis and nerve dysfunction occurring during lepromatous leprosy. Localization in erector pili muscles and hair follicles may explain the frequent occurrence of inflammation around these structures during secondary syphilis with the clinical manifestation of alopecia, including "motheaten" hair loss on the scalp and loss of the lateral third of the eyelashes.

A major goal of this ongoing study is to determine the fate of *T pallidum* inoculated into "immune" animals. Specific immunofluorescent localization of *T pallidum* in sites of intradermal inoculation revealed intact organisms for the first 1–2 days; the subsequent decrease in numbers of organisms correlated with the appearance of mononuclear cells containing fragments of fluorescent material. Similar observations in primary testicular lesions are seen when organisms are being phagocytized and digested by macrophages.[9] Electron-microscopic studies, including immunolabeling methods for *T pallidum* now being developed, are underway; and the results should clarify further the questions raised by these experiments.

References

1. Chesney AM: Immunity in syphilis. Medicine 1926, 5:463–547
2. Turner TB, Hollander DH: Biology of the treponematoses. WHO Monogr Ser 1957, 1–278
3. Sell S, Norris S: The biology, pathology and immunology of syphilis. Int Rev Exp Pathol 1983, 24:203–276
4. Baker-Zander S, Sell S: A histopathologic and immunologic study of the course of syphilis in the experimentally infected rabbit: Demonstration of long-lasting cellular immunity. Am J Pathol 1980, 101:387–414
5. Lukehart SA, Baker-Zander SA, Lloyd RMC, Sell S: Characterization of lymphocyte responsiveness in early experimental syphilis: II. Nature of cellular infiltration and *Treponema pallidum* distribution in testicular lesions. J Immunol 1980, 124:461–467
6. Sell S, Gamboa D, Baker-Zander SA, Lukehart SA, Miller JN: Host response to *Treponema pallidum* in intradermally-infected rabbits: Evidence for persistence of infection at local and distant sites. J Invest Dermatol 1980, 75:470–475
7. Lukehart SA, Baker-Zander SA, Sell S: Characterization of lymphocyte responsiveness in early syphilis I *in vitro* response to mitogens and *Treponema pallidum* antigens. J Immunol 1980, 124:454–460
8. Sell S, Baker-Zander SA, Lloyd RMC: T cell hyperplasia of lymphoid tissues in rabbits infected with *T pallidum*: A preliminary report showing no evidence for immune suppression. Sex Trans Dis 1980, 7:34–44
9. Sell S, Baker-Zander S, Powell HC: Experimental syphilitic orchitis in rabbits: Ultrastructural appearance of *Treponema pallidum* during phagocytosis and destruction by macrophages *in vivo*. Lab Invest 1982, 46:355–364
10. Collart P, Franceschini P, Durel P: Experimental rabbit syphilis. Brit J Vener Dis 1971, 47:389–401
11. Magnuson HJ, Rosenau BJ: The rate of development and degree of acquired immunity in experimental syphilis. Am J Syph 1948, 32:418–436

12. Medici MA: The immunoprotective niche—A new pathogenic mechanism for syphilis, the systemic mycoses, and other infectious diseases. J Theor Biol 1972, 36:617-625
13. Magnuson HJ, Eagle H, and Fleischman R: The minimal infectious inoculum of *Spirochaeta pallida* (Nichols strain) and a consideration of its rate of multiplication *in vivo*. Am J Syph 1948, 32:1-18
14. Magnuson HJ, Thomas EW, Olansky S, Kaplan BI, de Mello L, Cutler JC: Inoculation of syphilis in human volunteers. Medicine (Baltimore) 1953, 35:33-82
15. Bishop NH, Miller JN: Humoral immune mechanisms in acquired syphilis, Pathogenesis and Immunology of Treponemal Infection. Edited by RF Schell, DM Musher. New York, Marcel Dekker, 1983, pp 241-269
16. Ovcinnikov NM, Delektorskij VV: Ultrafine structure of the cell elements in hard chancres of the rabbit and their interrelationship with *Treponema pallidum*. Bull WHO 1970, 42:437-444
17. Ovcinnikov NM, Delektorskij VV: Current concepts of the morphology and biology of *Treponema pallidum* baised on electron microscopy. Br J Vener Dis 1972, 47:315-328
18. Azar HA, Pham TD, Kurban AK: An electron microscopic study of a syphilitic chancre. Arch Pathol 1970, 90:143-150
19. Sykes TA, Miller JN: Intracellular location of *Treponema pallidum* (Nichols strain) in the rabbit testes. Infect Immun 1971, 4:307-314
20. Lauderdale V, Goldman JN: Serial ultrathin sectioning demonstrating the intracellularity of *T pallidum*: An electron microscopic study. Br J Vener Dis 1972, 48:87-96
21. Sykes JA, Miller JN, Kalan AJ: *Treponema pallidum* within cells of a primary chancre from a human female. Br J Vener Dis 1974, 50:40-44
22. Sykes JA, Kalan AJ: *Treponema pallidum* in cells of a syphilitic lesion of the uterine cervix. Am J Obstet Gynecol 1975, 122:361-367
23. Ovcinnikov NM, Delektorskij VV: *Treponema pallidum* in peripheral nerves of rabbit syphiloma. Vest N Dermatol Venerol 1975, 5:38-40
24. Ovcinnikov NM, Delektorskij VV: *Treponema pallidum* in nerve fibers. Br J Vener Dis 1975, 51:10-18
25. Johnson RT: The pathogenesis of herpes virus encephalitis: I. Virus pathways to the nervous system of suckling mice demonstrated by fluorescent antibody staining. J Exp Med 1964, 119:343-365
26. Knotts FB, Cook ML, Stevens JG: Latent herpes simplex virus in the central nervous system of rabbits and mice. J Exp Med 1973, 138:740-744
27. Cook ML, Bastone VB, Stevens JG: Evidence that neurons harbor latent herpes simplex virus. Infect Immun 1974, 9:946-951